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(54) Title: TELOMERE–ASSOCIATED CHROMOSOME FRAGMENTATION

(57) Abstract

The present invention relates to telomere–associated chromosome fragmentation by the use of synthetic telomeric DNA constructs comprising a telomeric region, a selectable marker gene and a homologous region, its application in a targeted manner to delete chromosomal regions in eukaryotic cells such as embryonic stem (ES) cells, and the construction of mice in which telomeric regions have been deleted or altered. It is demonstrated that the murine immunoglobulin heavy chain locus located at the telomeric end of chromosome (12) can be entirely deleted by targeted chromosome fragmentation in ES cells using a construct incorporating a human cloned telomeric fragment, and that mice can thereby be produced in which this locus, which is essential for antibody production, is missing.

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TELOMERE-ASSOCIATED CHROMOSOME FRAGMENTATION

The present invention relates to telomere-associated chromosome fragmentation by the use of synthetic telomeric DNA constructs, and the construction of non-human animals such as mice in which telomeric regions have been deleted or altered.

BACKGROUND TO THE INVENTION

A telomere is a DNA sequence element which is needed at each end of a linear chromosome and which is essential for stable chromosome maintenance . Replication on the lagging strand of a replication fork requires the presence of some DNA ahead of the sequence to be copied that can serve as the template for an RNA primer. Since there can never be such a template for the last few nucleotides of a linear DNA molecule, each such DNA strand would become shorter with each replication cycle. Eukaryotic cells have evolved a special telomeric DNA sequence at each chromosome end. This simple repeating sequence is periodically extended by a special enzyme, telomerase, thus compensating for the loss of a few nucleotides of telomeric DNA in each cycle and permitting a linear chromosome to be completely replicated.

The molecular characterisation of telomeres began in the 1970s with the identification of tandemly repeated simple sequences at the ends of ciliate chromosomes. It proceeded with the identification of similar repeated sequences in yeast chromosomal telomeres and the demonstration that these sequences were sufficient to stabilise linear chromosomes. In the 1980s, hypervariable telomeric sequences were obtained from human sex chromosomes. (See for example Moyzis *et al*, Proc. Nat. Acad. Sci. USA 1988, Vol. 85, pp 6622-6626).

Farr *et al* in Nature Genetics, Vol. 2, December 1992, pp 275-282 described telomere-associated chromosome fragmentation (TACF) as a new approach for chromosome mapping based on the non-targeted introduction of cloned telomeres into mammalian cells. A cloned human telomeric fragment was used to generate a panel of somatic cell hybrids with nested terminal deletions of the long arm of human X chromosome.

Itzhaki *et al* in Nature Genetics, Vol. 2, December 1992, pp 283-287 disclosed the targeted breakage of a human chromosome mediated by cloned human telomeric DNA. They described the combined use of gene targeting and telomere-mediated chromosome breakage to generate a defined truncation of a human chromosome. Telomeric DNA was targeted to the 6-16 gene on the short arm of chromosome 1 in a human cell line. Molecular and cytogenic analyses showed that, of eight targeted clones isolated, one clone had the predicted truncation of chromosome 1. The results appear to indicate deletion of a fragment of approximately 30 megabase-pairs from the tip of the short arm of chromosome 1: this fragment including several exons from the 6-16 gene locus.

Barnett *et al* in Nucleic Acids Research 1993, Vol. 21, No. 1, pp 27-36 also discussed telomere directed fragmentation of mammalian chromosomes. It was observed that cloned human telomeric DNA could integrate into mammalian chromosomes and seed the formation of new telomeres. The cytogenic consequences of the seeding of new telomeres included large chromosome truncations but most of the telomere seeding events occurred close to the pre-existing ends of natural chromosomes. Barnett *et al* suggested that truncated chromosomes, referred to as mini-chromosomes, could possibly be made which were small enough to be shuttled into an experimental environment where they could be analysed and manipulated more easily than in a mammalian cell: the example given being the hypothetical introduction of a mini-chromosome into the nucleus of *S. cerevisiae*. It was also suggested that if the mini-chromosome could be reintroduced into a mammalian cell and retain its integrity then it could become the basis of a mammalian artificial chromosome vector. The actual introduction of a mini-chromosome as a vector into another hose cell was not disclosed.

In a review article in Current Opinion in Genetics and Development, 1994, Vol. 4, pp 203-211 Greider discussed chromosome fragmentation in general terms, including a discussion of spontaneous breakage and healing, random chromosome fragmentation, and targeted chromosome fragmentation. As stated in the abstract to the review, telomeres have been used to specifically fragment mammalian chromosomes to detect their structure and function.

Clearly an understanding of mammalian telomere dynamics provides insight into the normal and abnormal functioning of endogenous mammalian chromosomes.

BRIEF DESCRIPTION OF THE INVENTION

It has now surprisingly been found according to the present invention that a cloned telomeric fragment can be produced which can be integrated by homologous integration into a chromosome and can produce a targeted deletion of an entire chromosomal locus. In contrast to previous work which may (or may not) have resulted in some effect on chromosomal integrity by deletion of fragments of a chromosomal locus, the present invention ensures the total and complete absence of an entire chromosomal locus by its complete deletion from the chromosome.

Further, it has been surprisingly found that according to the invention a human cloned telomeric fragment can be used to delete an entire chromosomal locus from a eukaryotic chromosome, such as a mouse chromosome. This creates a novel locus-deleted, truncated, chimeric chromosome.

More particularly, it has surprisingly been found according to the present invention that a human telomeric construct including a region homologous to a section of the mouse chromosome 12 downstream and separated from a specific locus can be homologously integrated in order to result in complete deletion of the specified locus. More specifically, the present invention provides a human telomeric construct including a homologous region of mouse chromosome 12 and capable of complete deletion of the entire mouse IgH locus. Following transfection of this construct into mouse ES cells and successful homologous integration, ES cells with a deleted mouse IgH allele can be identified and used to obtain chimeric mice which can be bred to result in the germline transmission of the characteristic of complete inability to produce the immunoglobulin heavy chain.

Those skilled in the art will appreciate that the same technique could be used to provide a telomeric construct capable of complete deletion of a different chosen locus or multiple chosen loci in the same or different eukaryotic cells.

Having understood the general principle of the invention, it will be appreciated by those skilled in the art that it would also be possible to modify a telomeric construct in order that it should include at least one functional gene capable of expressing a gene product and that such a gene could consequently be carried into the modified chromosome at the same time as homologous recombination occurs. For example, it is preferred according to the invention to include in the telomeric construct a selectable marker gene which would allow identification of correctly recombined products. It will be appreciated that as well as or instead of a selectable marker gene another DNA sequence containing a gene encoding a gene product could be included in the construct at a suitable position between the telomeric region and the homology region. Thus, by way of example, the present invention could provide a cloned telomeric fragment, including in the correct genomic orientation, a telomeric region, a selectable marker gene, an exogenous functional gene, and a homologous region. More specifically, in the embodiment wherein a human telomeric region and a selectable marker and a homologous region of mouse chromosome 12 are provided capable of complete deletion of mouse IgH locus, it is within the scope of the present invention to include in the construct between the telomeric region and the homology region a complete human IgH locus or a gene(s) encoding part of the locus. If such a construct were integrated into ES cells by homologous integration it would be possible to select ES cells with a deleted mouse IgH allele and an inserted human IgH allele (or part thereof) for use in obtaining chimeric mice completely incapable of producing endogenous immunoglobulin heavy chains but capable of producing exogenous human immunoglobulin heavy chains (or parts thereof).

Although the present invention is illustrated herein by an example involving the entire deletion of the murine IgH locus, it is to be understood that possible deletions/insertions could involve (as well or instead) the immunoglobulin light chain locus or other loci of interest.

FIGURES

The invention will now be described by way of example only with reference to the following figures in which:

Figure 1 shows a region of mouse chromosome 12 downstream from Cα of the IgH locus (marked "IgH locus") and also shows a construct (marked "ΔIgH locus") in which a homologous mouse region is attached to a selectable neo^r marker and a telomere, the construct being capable of homologous integration and deletion of the entire IgH locus.

Figure 2 shows the analysis of Southern blots of ES cell DNA following homologous integration. In the upper panel, a μ specific probe was used and gives half the signal of the control C when deletion has occurred. In the lower panel, probe A of Figure 1 was used; smearing is due to size heterogeneity of the new telomeric end (region indicated).

Figure 3 shows the process of telomere fragmentation/replacement in which the original full length chromosome (top bar) including the IgH locus and the endogenous telomere is homologously integrated with a construct (middle short bar) comprising an exogenous telomere, a selectable marker region, and a homologous region to provide a chimeric shortened chromosome (lower bar) having the exogenous telomere and selectable marker but a completely deleted IgH locus.

Figure 4 shows FISH analysis of normal mouse ES cells and ES cells after telomere deletion, both probed with fluorescent, heavy chain specific probes which hybridise with the telomeric region of chromosome 12. In the centre of the Figure 4A there is a chromosome spread of a control using normal ES cells giving two doublet signals from two chromosomes. In the centre of the Figure 4B there is a chromosome spread of cells of the invention. Such telomere-deleted ES cells give only one doublet signal from only one chromosome.

Figure 5 shows the result of a PCR (polymerase chain reaction) carried out on mice in which germline transmission of ES cells carrying the targeted deletion of the IgH locus had been achieved. The PCR analysis was performed using primers specific for the neomycin

resistance (neo^r) gene, which is part of the telomeric construct used to perform the fragmentation on chromosome 12. Markers at the ends of the gel are 100bp ladders. From the left, track 1 = Marker, track 2 = PCR reaction with no DNA; track 3 = normal mouse DNA; track 4 = unrelated transgenic mouse DNA with neo gene (positive control); tracks 5 - 13 = germline transmission mice analysed for neo gene by PCR, showing that mice 1, 5, 7, 8, 9 are positive for the construct; track 14 = Marker.

Example 1: Telomere-directed fragmentation of mouse chromosome 12

Background

The mouse immunoglobulin heavy chain locus is located at the distal end of chromosome 12. The orientation is telomere-V1-V2-Vn-D-J-Cμ-Cγ-Cα. By homologous recombination it is possible to direct telomere integration to specific sites which result in deletion of the entire IgH locus.

Experimental design and procedures

The example provides a DNA construct in order to delete the IgH locus by telomere seeding or fragmentation. The construct comprises a 7.5kb region (the homology region) separated from and downstream of Cα (the last C region in the heavy chain locus) isolated from a 129 mouse ES cell library (gift of Dr T. Rabbitts and Dr. A Smith), linked to to a ~0.8kb human telomere region (gift of Dr. C. Farr) duplicated to 1.6kb and a selectable marker gene for neomycin resistance, neo^r. This construct was cloned into the pUC plasmid and linearised. Following transfection of this construct into ZX3 ES cells (unpublished) homologous integration was identified by PCR and enzyme digestion, Southern blotting and FISH analysis. ES cells with a deleted IgH allele were then used to obtain chimeric mice.

A construct was obtained that links the mouse IgH 3' enhancer region to a selectable marker gene, namely neomycin resistance (neo^r) and the new human-derived telomere (Figure 1).

The new telomere comprised cloned human telomeric DNA. It is preferred according to the invention that the telomere is elongated as this appears to give better results. In the present example the telomeric region used is essentially a duplex of an 800bp repetitive region. This 2x800bp region is provided on a Not 1 fragment and is prepared by duplicating through ligation and subcloning.

As can be seen from Figure 1, the homologous region contained an endogenous 3' enhancer. This homologous region was approximately 20kb downstream of the IgH locus. It is not necessary to know precisely the separation between the IgH locus and the homology region, but it is essential that there is a separation between the two in order to ensure that complete deletion of the entire IgH locus is achieved on homologous integration.

This construct was transferred into embryonic stem (ES) cells by electroporation. In two approaches about 40 G418 resistant clones were obtained and analysed by Southern blot, PCR and FISH (fluorescence in situ hybridisation). The rather low number of ES cell clones and the initial Southern blot hybridisation indicated that integration of telomeric DNA leads frequently to chromosome fragmentation and subsequent cell death. Therefore random integration is to some extent avoided and site-specific integration appears to be dominant. As shown in Figure 2 (upper) when roughly equal amount of DNA from ES cell clones were hybridised with a 5' IgH region probe (for example Cμ) control DNA (C) from normal ES cells shows an increased signal, indicating deletion of one copy of the IgH locus in the targeted cell.

The evidence that the IgH locus was deleted in ES cell clones is derived from the results of both Southern blot analysis and FISH analysis. Southern blotting, as represented by Figure 2, showed in a number of clones 1) a fainter hybridisation signal for the IgH locus than that given by control (C) ES cells (Figure 2 top); 2) the presence of the targeting vector, 3) the presence of a diffuse 5' vector integration signal due to imprecise telomere elongation (Figure 2 bottom). In a separate Southern blot (not shown) probe B was used on a HindIII digest of DNA from selected transformed ES clones (see Figure 2) and showed that in those clones which had integrated the construct, the germline configuration of the HindIII fragment shown in Figure 1 was maintained. Since random integration yielded a different HindIII fragment, this result implies that integration is site-specific in those selected clones.

Further evidence was obtained that the neo^r gene was incorporated at the telomere. Thus in the selected clones above a neo^r fragment could be obtained by PCR on extracted DNA, but

after short-term exonuclease digestion of the DNA with Bal31, the neo^r gene was no longer recoverable, and by implication located close to the free end of a chromosome.

FISH analysis using an IgH region probe was carried out by established methods. The results in Figure 4 indicate that ES cell clones with deleted IgH locus have been obtained. Thus whereas in control ES cells two doublet signals were obtained when probed with flurorescent heavy chain specific probes (Figure 4A), in the ES cell clones treated with the telomeric construct, only one doublet signal is seen (Figure 4B).

The ES cells described above were injected into BALB/c blastocysts and introduced into CD1 foster mothers, leading to production of chimaeric offspring as recognised by coat colour. The chimaeric males were used for breeding to BALB/c females, resulting in germline transmission. This was demonstrated by PCR of tail-extracted DNA using primers specific for the neo^r gene which is part of the targeting construct (Figure 5). As shown, 5 out of 9 mice (Nos. 1,5,7,8,9) tested carried the homologous integration event, implying deletion of the endogenous telomere, since the ES cells carried this deletion as a result of the integration of the construct. Southern blotting with a probe for a 3' region of the mouse IgH locus, present normally on chromosome 12, confirmed that mice 1, 5, 7, 8, 9 carried the deletion phenotype.

These heterozygous germline transmission mice are suitable for breeding of the chromosome deletion to homozygosity. Homozygous mice carrying such a deletion would be incapable of producing immunoglobulins. They would therefore be suitable animals into which transgenes carrying the human immunoglobulin regions could be intoduced. It has been shown that efficient production of human antibodies in transgenic mice requires that the endogenous mouse heavy chain locus be silenced. Thus, the present invention of telomeric fragmentation of the mouse IgH locus provides a novel strain of 'heavy chain knockout' mouse suitable for the production of human antibodies.

CLAIMS

1. A method for producing a targeted deletion of a eukaryotic genetic locus or part of a locus, which method comprises homologous integration into a chromosome of a targeting vector carrying a cloned telomeric fragment, such that a locus-deleted, truncated or altered chromosome is obtained.

2. A method in which any genomic region is deleted, or partially deleted, according to claim 1.

3. A method according to claim 1 in which the targeting vector carries a cloned telomeric fragment and also a selectable marker gene such as a neomycin resistance gene or other selectable gene.

4. A targeting vector for carrying out targeted deletion of part of a eukaryotic chromosome, by the method according to claim 1, the said vector carrying another genomic region in addition to the selectable marker.

5. A targeting vector according to claim 4 in which the additional genomic region is homologous to a nucleotide sequence on the targeted chromosome.

6. A targeting vector for carrying out targeted deletion of part of a eukaryotic chromosome, by the method according to claim 1, the said vector carrying a telomeric region, a selectable marker gene such as a neomycin resistance or other selectable gene, an exogenous functional gene and a homologous region to allow site-specific fragmentation and integration.

7. A method according to claim 1 in which the targeted chromosome carries the locus specifying immunoglobulin heavy chains, such as chromosome 12 in the mouse, and the product obtained by the method is a chromosome from which all or part of the heavy chain locus is deleted.

8. A method according to claim 1 in which targeted deletion is performed in embryonic stem (ES) cells.

9. A method whereby the ES cells produced as a result of carrying out the method of claim 8 are used to generate chimeric animals and/or germline transmission animals.

10. A method in which animals generated according to claim 9 are subject to breeding, to produce animals which are homozygous for the altered chromosome.

11. Animals according to claim 10 in which deletion of the chromosomal region is present in all respective alleles.

12. Animals according to claims 9 - 11 which are unable to produce the gene products of the deleted or partly deleted locus.

13. Animals according to claim 10 which are unable to produce endogenous immunoglobulin heavy chains.

14. Animals according to claim 13 into which transgenes carrying heterologous immunoglobulin genes or gene loci have been introduced.

15. Animals according to claim 14 in which the immunoglobulin transgenes are human.

16. Animals according to claim 15 which express human immunoglobulins and which respond to immunisation with antigens by production of human antibodies.

17. Hybridomas and human monoclonal antibodies produced from animals according to claim 16.

18. A method for the production of mice in which all or part of the immunoglobulin heavy chain (IgH) locus has been deleted, comprising

(a) introduction into embryonic stem (ES) cells of a targeting vector containing a telomeric region, a selectable marker and a mouse homology region, such that replacement of the telomeric region of chromosome 12 through homologous integration of the targeting vector occurs;
(b) identification of the modified ES cells
(c) introduction of the modified ES cells into blastocysts in order obtain chimeric animals
(d) obtaining germline transmission of the altered chromosome 12
(e) breeding of animals carrying the altered chromosome 12 to homozygosity

19. Mice produced according to the method of claim 18

20. A method for the production of transgenic mice capable of producing human immunoglobulins, comprising
(a) deletion of the mouse IgH locus by the method of claim 18
(b) introduction of human Ig heavy and/or light chain transgenes by breeding or by use of ES cells in which the mouse Ig loci have been deleted by the method of claim 18

21. Transgenic mice produced according to the method of claim 20.

22. A method for the targeted deletion of a variable part of a chromosome, comprising
(a) introduction into ES cells of a targeting vector containing a telomeric region, a selectable marker and a homology region, such that replacement of part of a chromosome through homologous integration of the targeting vector occurs, and
(b) identifying the modified ES cells, and optionally collecting the modified ES cells.

23. A method for obtaining animals in which a variable part of a chromosome has been deleted, comprising
(a) introduction of the modified ES cells made as in claim 22 into blastocysts in order to obtain chimeric animals
(b) obtaining germline transmission of the altered chromosome carrying the new telomere
(c) breeding of animals carrying the altered chromosome to homozygosity.

24. A method for the introduction of a genetic region into a chromosome, comprising
(a) introduction into ES cells of a targeting vector containing a telomeric region, a selectable marker, a homology region and the specified genetic region,
(b) identifying the modified ES cells, an optionally collecting the modified ES cells.

25. A method for obtaining animals in which a genetic region has been introduced into a chromosome, comprising
(a) introduction of the modified ES cells produced according to claim 24 into blastocysts in order obtain chimeric animals,
(b) obtaining germline transmission of the altered chromosome carrying the new telomere.

26. A method according to claims 24 and 25 in which the animals are mice, and in which the chromosome region deleted includes the immunoglobulin heavy chain locus and the genetic region introduced carries the human immunoglobulin heavy or light chain locus.

27. Hybridomas and monoclonal antibodies produced by contacting animals, made by using any of the preceding method claims, with antigen.

28. Modified ES cells made by using any of the preceding method claims.

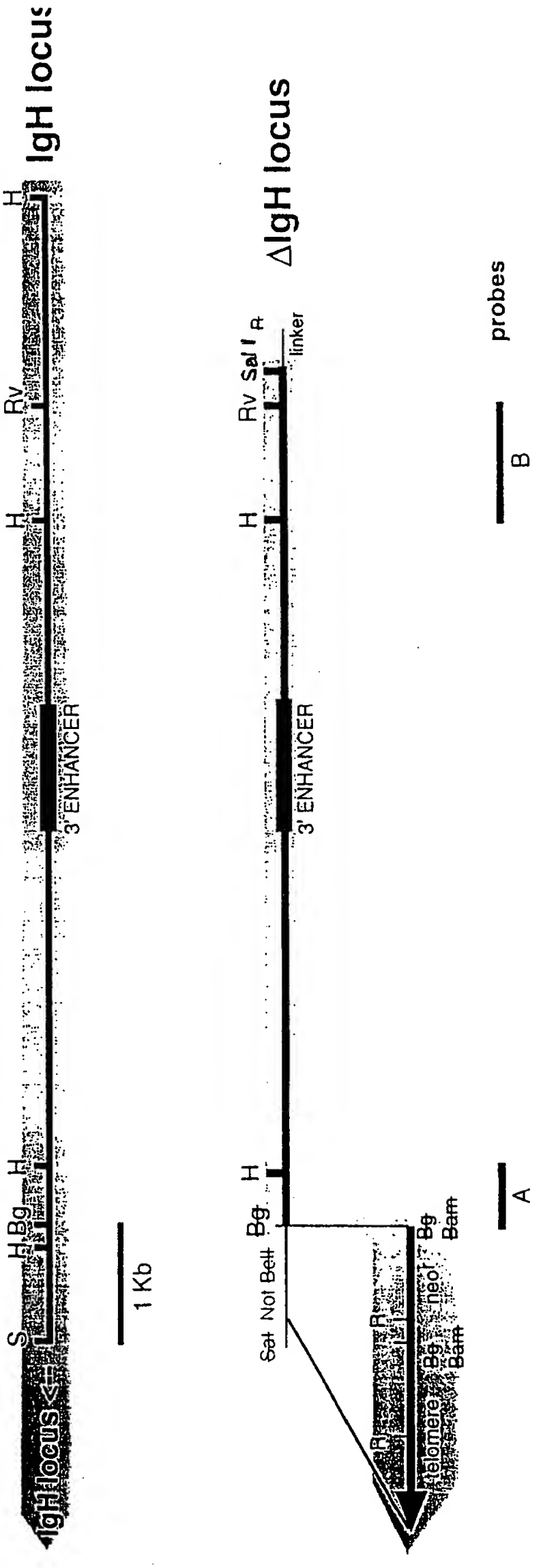

IgH locus
H
Rv
H
3' ENHANCER
S
H Bg H
IgH locus
1 Kb
ΔIgH locus
H
Rv Sal I R
linker
3' ENHANCER
H
Bg
Sal Not Bell
R
neo
Bg
Bam
Telomere
Bg
Bam
A
B
probes
Hind III (H) digestion probe A germline band ~ 0.7 Kb
targeting band ~ 3.1 Kb (poss 4.5 Kb)
depending on telomere elongation
Hind III (H) digestion probe B germline band ~ 3 Kb
targeting band ~ 3 Kb

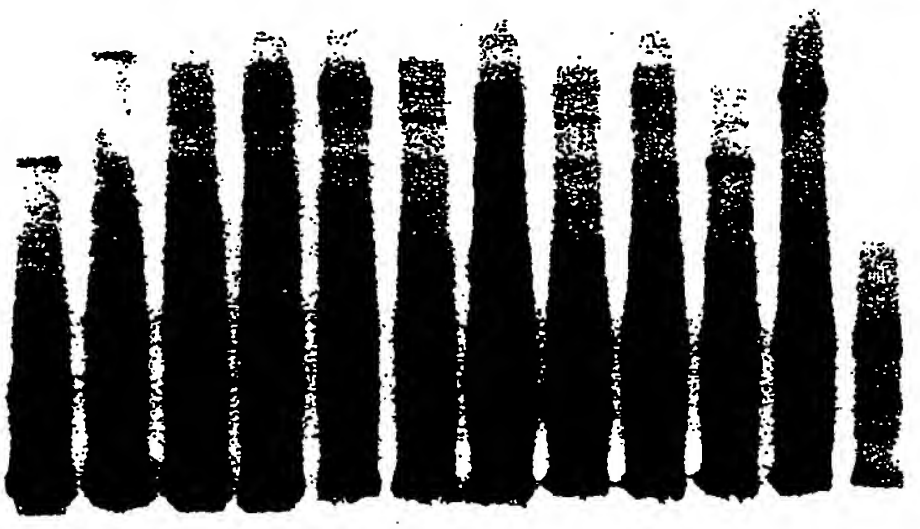
FIG. 1

HindIII digested DNA hybridised with a 5' IgH probe

C C X4 C X5 C X8 C X14 C X15 X17
1/2 1/2 1/3

The loaded amounts of DNA are roughly identical (confirmed by optical density and ethidium bromide staining) except were stated for controls. Clones which clearly show a reduced signal are X4. X8. X14. X15 and X17 but not X5.

HindIII digested DNA hybridised with probe A



The hybridisation probe highlights a band which shows the released telomere with a single HindIII site.

FIG 2

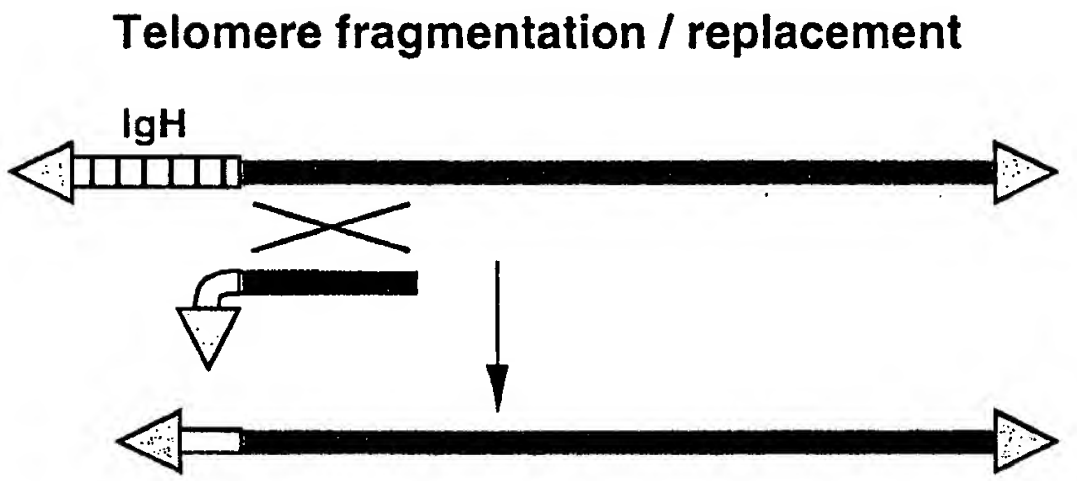


FIG 3

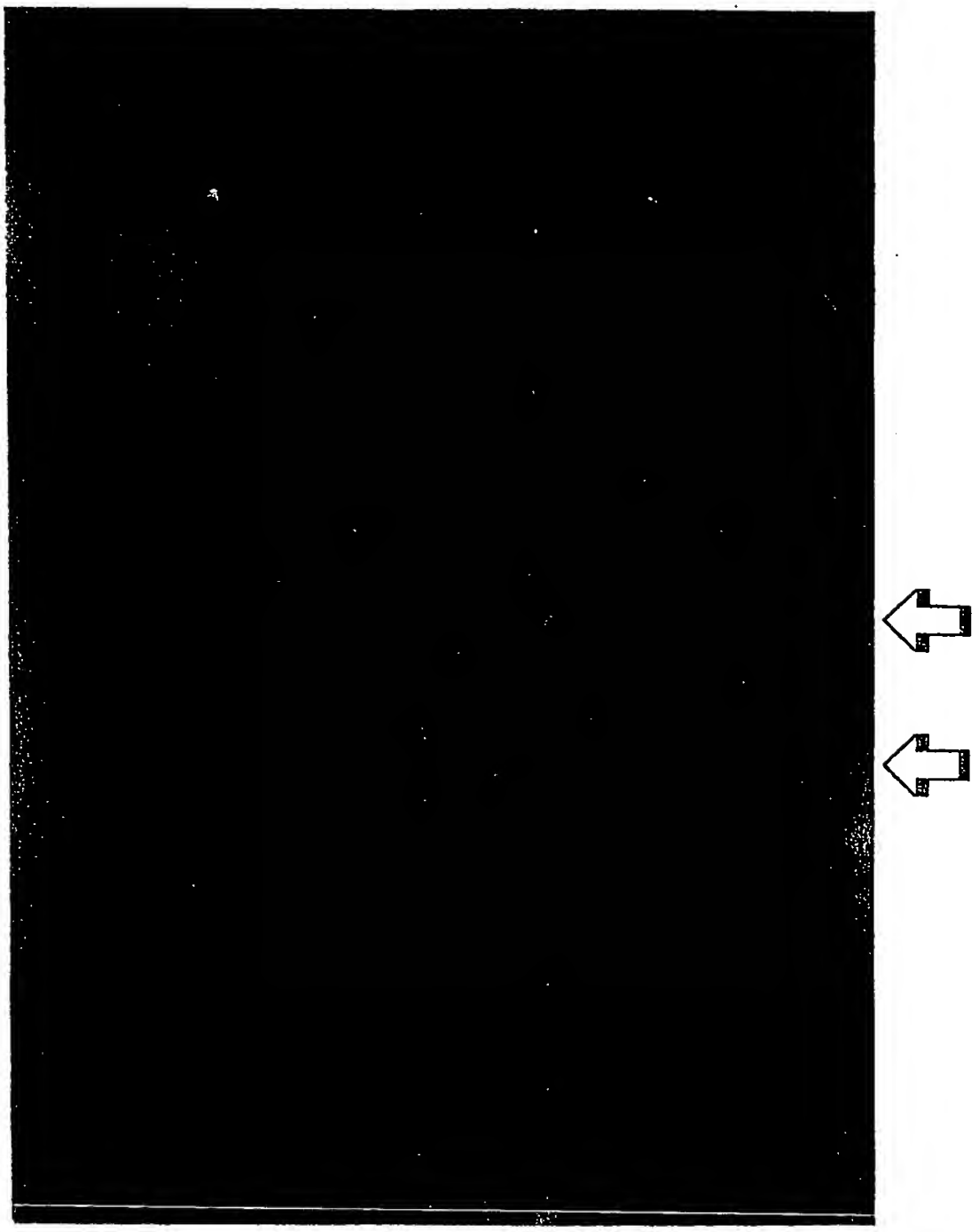


FIG 4A

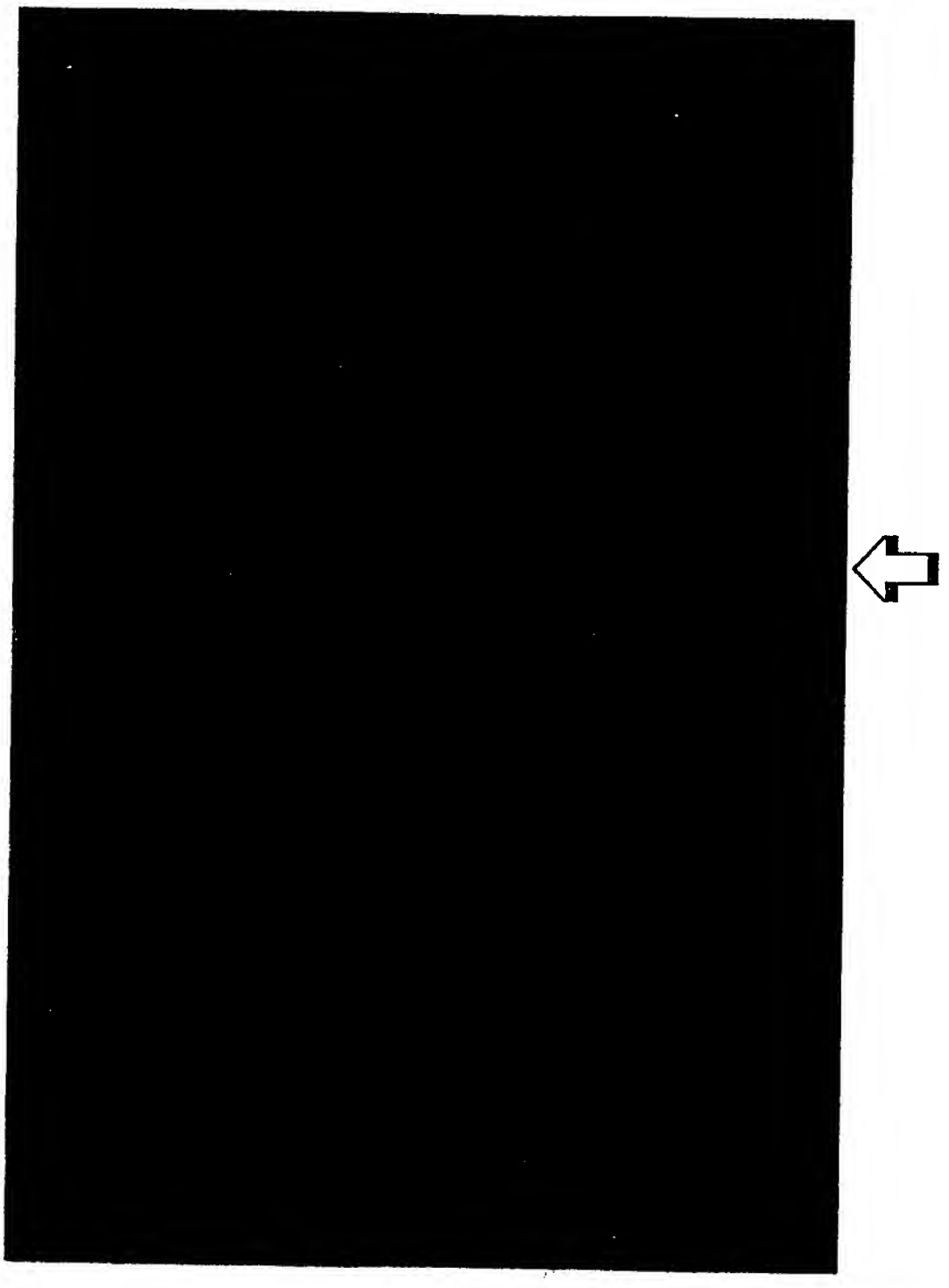


FIG 4B

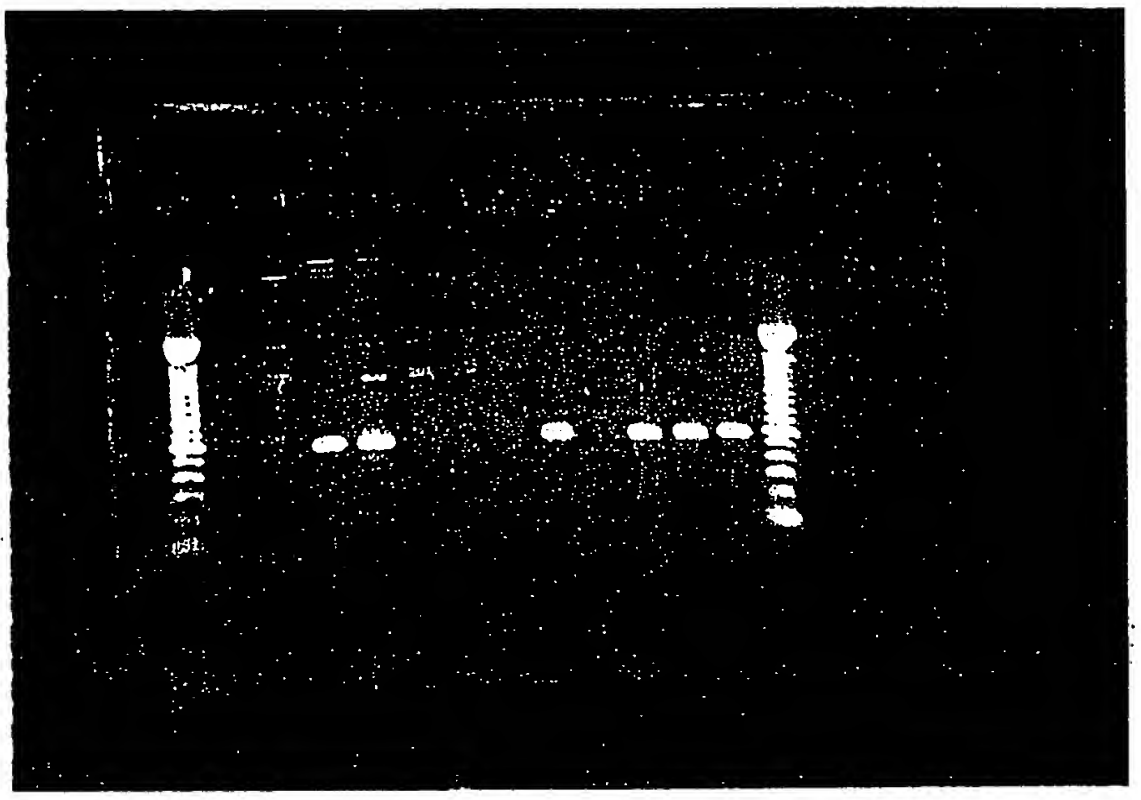


Fig 5

INTERNATIONAL SEARCH REPORT

Inte .ional Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/90 A01K67/027 A61K48/00 C12N5/12 C12N15/12
C07K16/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practical. search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
Y	C. FARR ET AL.: "Generation of a human X-derived minichromosome using telomere-associated chromosome fragmentation." THE EMBO JOURNAL, vol. 14, no. 21. 1 November 1995, pages 5444-5454, XP002081575 Oxford, GB see abstract see figure 1 ---	1-28
Y	WO 94 25585 A (GENPHARM INTERNATIONAL, INC.) 10 November 1994 see examples see claims --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropnate, of the relevant passages	Relevant to claim No.
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